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## Research Note

# Subtyping *Listeria monocytogenes* from Bulk Tank Milk Using Automated Repetitive Element–Based PCR†

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## ABSTRACT

Sixty-one *Listeria monocytogenes* strains from raw milk were analyzed with an automated repetitive element–based PCR (rep-PCR) system to examine the utility of this system for serotype grouping and to determine whether specific regional relationships could be identified. Results of the similarity analysis revealed two primary clusters of *L. monocytogenes* isolates. Cluster 2 exclusively contained serogroup 1/2a isolates; however, two 1/2a isolates were also found in cluster 1. Isolates of serogroups 1/2b, 4b, 3b, and 4c were also in cluster 1. Clusters 1 and 2 were separated at a relative similarity of 86%. *Listeria* species other than *L. monocytogenes* (*L. ivanovii, L. seeligeri, L. welshimeri, L. grayi,* and *L. innocua*) had similarity scores of less than 80% in pairwise comparisons with the *L. monocytogenes* isolates. Thus, this method may be useful for species identification once an isolate is characterized as *Listeria.* When rep-PCR fingerprints of the *L. monocytogenes* 1/2a isolates were compared, there was no apparent regional grouping. However, discrimination between isolates suggests that the rep-PCR assay might be useful for tracking *L. monocytogenes* 1/2a and for tracking isolates across regions or within smaller ecological niches. The automated rep-PCR method could not discriminate between serotypes 1/2b and 4b but may be useful for discriminating between 1/2a and other serotypes and for tracking isolates within serotype 1/2a.

Listeriosis is a foodborne disease caused by *Listeria monocytogenes,* and this disease afflicts approximately 2,500 people in the United States each year *(10). L. monocytogenes* is also an animal pathogen that can cause encephalitis and abortion in cattle *(23)* and has occasionally been implicated as a causative agent of mastitis in dairy cows *(7, 17).* As in humans, *L. monocytogenes* carriage can be asymptomatic in cattle, and a herd may be unknowingly infected *(23). L. monocytogenes* contamination of raw milk is not uncommon *(4, 6, 11, 16, 19, 21).*

*L. monocytogenes* isolates traditionally have been serotyped to distinguish between isolates or to identify related outbreaks and sources. Thirteen serovars of *L. monocytogenes* have been described *(2),* but only a few of these (1/2a, 1/2b, and 4b) are commonly associated with infected humans *(20).* Serotype 4b is implicated in most epidemic outbreaks, and serotypes 1/2a and 1/2b are typically identified in sporadic cases of listeriosis *(18).* Serotyping is an expensive and time-consuming process, and for many epidemiological studies this method of classifying *L. monocytogenes* isolates does not appear to have sufficient discriminatory power. In recent years, DNA-based methodologies such as repetitive sequence–based PCR (rep-PCR) have been developed to differentiate bacterial isolates *(12).* The rep-PCR assay appears to have sufficient discriminatory power to trace outbreaks of foodborne illnesses such as listeriosis *(8, 9, 12).*

In a recent survey of raw bulk tank milk, we isolated *L. monocytogenes* from 58 dairy farms across the United States *(21).* More than 90% of the isolates were of serotypes 1/2a, 1/2b, or 4b. Regional differences in prevalence were observed, and there also appeared to be regional variations in serotype distribution of the milk sample isolates. However, serotyping alone did not delineate sufficient differences between the isolates to determine potential relatedness within regions.

The objectives of this study were to evaluate the use of an automated rep-PCR system to classify the *L. monocytogenes* isolates into serogroups and to determine whether more specific regional relationships could be identified within the serogroups.

## MATERIALS AND METHODS

The 61 *L. monocytogenes* isolates used in the study were obtained from raw milk samples collected during the National Animal Health Monitoring System Dairy 2002 survey *(21).* Bulk tank milk samples were obtained from 861 farms in 21 states and sent to the U.S. Department of Agriculture, Agricultural Research Service (Beltsville, Md.). Processing, detection, and isolation procedures were described previously *(21).* Serotyping of the isolates characterized as *L. monocytogenes* was conducted with a previ-

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ously described enzyme-linked immunosorbent assay *(13).* This method includes a quantitative colorimetric reaction to score positive antigen and antiserum reactions, thereby eliminating the subjective visual evaluation of the reaction.

Strains of *Listeria ivanovii, Listeria innocua, Listeria welshimeri, Listeria seeligeri,* and *Listeria grayi* were obtained from MicroBioLogics Inc. (Saint Cloud, Minn.) and were grown on tryptic soy agar with yeast extract. Genomic DNA was extracted from *Listeria* cultures with an UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, Calif.) according to the manufacturer's instructions. The DNA preparations were stored at −20°C until analysis. An automated microbial fingerprinting system (DiversiLab System, Spectral Genomics, Inc., Houston, Tex.) was used to generate DNA fingerprints of each isolate. This system incorporates microfluidics chips and an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, Calif.) to separate and quantify amplified DNA products. A commercial kit (DiversiLab Listeria Kit, Bacterial Barcodes, Inc., Houston, Tex.) was used for performing rep-PCR with AmpliTaq polymerase (Applied Biosystems, Foster City, Calif.) and for running the microfluidics chips according to the manufacturer's instructions. DiversiLab software (version 2.1.66) was used to analyze and compare the resulting electropherograms. With this program, similarity scores were determined for all possible pairs of samples based on Pearson correlations, and the relationships were transcribed into dendrograms with the unweighted pair group method with arithmetic mean.

## RESULTS

When the results of the similarity analysis of the 61 *L. monocytogenes* isolates were plotted in a dendrogram, two primary clusters of isolates were revealed (Fig. 1). Cluster 1 was composed of 37 isolates: 2 from serogroup 1/2a, 19 from serogroup 1/2b, 13 from serogroup 4b, 2 from serogroup 3b, and 1 from serogroup 4c. Cluster 2 was composed of 24 isolates that were exclusively from serogroup 1/2a, although 2 isolates from this serogroup were also found in cluster 1.

Within cluster 1, all isolates had a similarity score of greater than 90%, and there was a very high degree of similarity between isolates 1 through 31. Isolates 32 through 37 fell into three small clusters that were distinct from the other isolates in this cluster. Clusters 1 and 2 were separated at a relative similarity of approximately 86%. The isolates in cluster 2 were separated into several distinct subclusters that had variable relative similarities, all above 90%.

Rep-PCR analysis also was run on several *Listeria* species other than *L. monocytogenes.* The resulting DNA fingerprint data were compared with those of representative isolates from clusters 1 and 2 (Fig. 2). *L. grayi* isolates were the most similar to *L. monocytogenes* isolates from clusters 1 and 2, and *L. ivanovii* and *L. seeligeri* were the least similar.

Based on the results shown in Figure 1, it appeared that this method of genetic characterization may have sufficient discriminatory power to distinguish between clonal groups of *L. monocytogenes* within the 1/2a serogroup. The isolates used in this study were coded based on region (west, midwest, southeast, northeast), and the dendrogram in Figure 3 reflects this coding. The majority of the 1/2a isolates were from the northeast region, and these isolates were dispersed among 9 or 10 clusters.

## DISCUSSION

Thirteen serotypes of *L. monocytogenes* have been identified *(2),* and there appears to be substantial genotypic diversity between isolates within individual serotypes *(1, 3, 8, 14, 25).* Molecular genotyping methods have been developed for epidemiological tracing of bacterial isolates, species differentiation, and a variety of other research purposes *(12).* Pulsed-field gel electrophoresis appears to be the most discriminatory of these methods, but this procedure is costly and can take at least 3 days to complete. Analysis using rep-PCR produces results more quickly and has substantial discriminatory power *(12).* Jersek et al. *(8)* demonstrated that rep-PCR fingerprints of human and animal *L. monocytogenes* isolates were different from those of food isolates. They also were able to distinguish among serotypes within each major cluster.

An automated rep-PCR system that removes some of the potential variation associated with operators and laboratories may be useful for determining relatedness of bacterial isolates from relatively small ecological sites (i.e., within a farm system) or for studying the distribution of clonal groups within a large geographic region. Healy et al. *(5)* found that this automated system was robust to a variety of changing conditions (e.g., operators, instruments, DNA concentrations, and laboratories). By using *Neisseria meningitidis,* Healy et al. found that the system yielded results equivalent to those obtained by manual rep-PCR.

Because serotyping is time-consuming and complex, we investigated the usefulness of an automated rep-PCR system for categorizing the milk-derived isolates of *L. monocytogenes* into serotypes and for discriminating among isolates within individual serogroups. The rep-PCR assay was able to distinguish serogroup 1/2a from the remaining serogroups (Fig. 1). However, isolates from serogroups 1/2b, 4b, 3b, and 4c primarily fell within one large cluster, and there was no distinct pattern of subclustering. The two groups clearly represent very different *L. monocytogenes* lineages, with less than 65% similarity between the two clusters.

At least two and sometimes three distinct lineages of *L. monocytogenes* have been identified *(1, 15, 22, 24, 25).* Borucki et al. *(1)* compared the genetic relationships between 24 isolates of *L. monocytogenes* based on hybridization patterns derived from a DNA microarray. Their results revealed two primary clusters, one composed of serogroups 1/2b, 4b, and 4c and the other composed of serogroups 1/2a and 1/2c. Ward et al. *(22)* developed an intraspecific phylogeny of *L. monocytogenes* based on *prfA* virulence gene cluster sequences. They identified three distinct lineages and found that serotype 4b strains were prevalent in both lineage 1, which was frequently associated with human clinical strains, and lineage 3, which was less frequently associated with human listeriosis strains.

Although most of the 1/2a isolates in this study were grouped in a single cluster, two indistinguishable 1/2a iso-

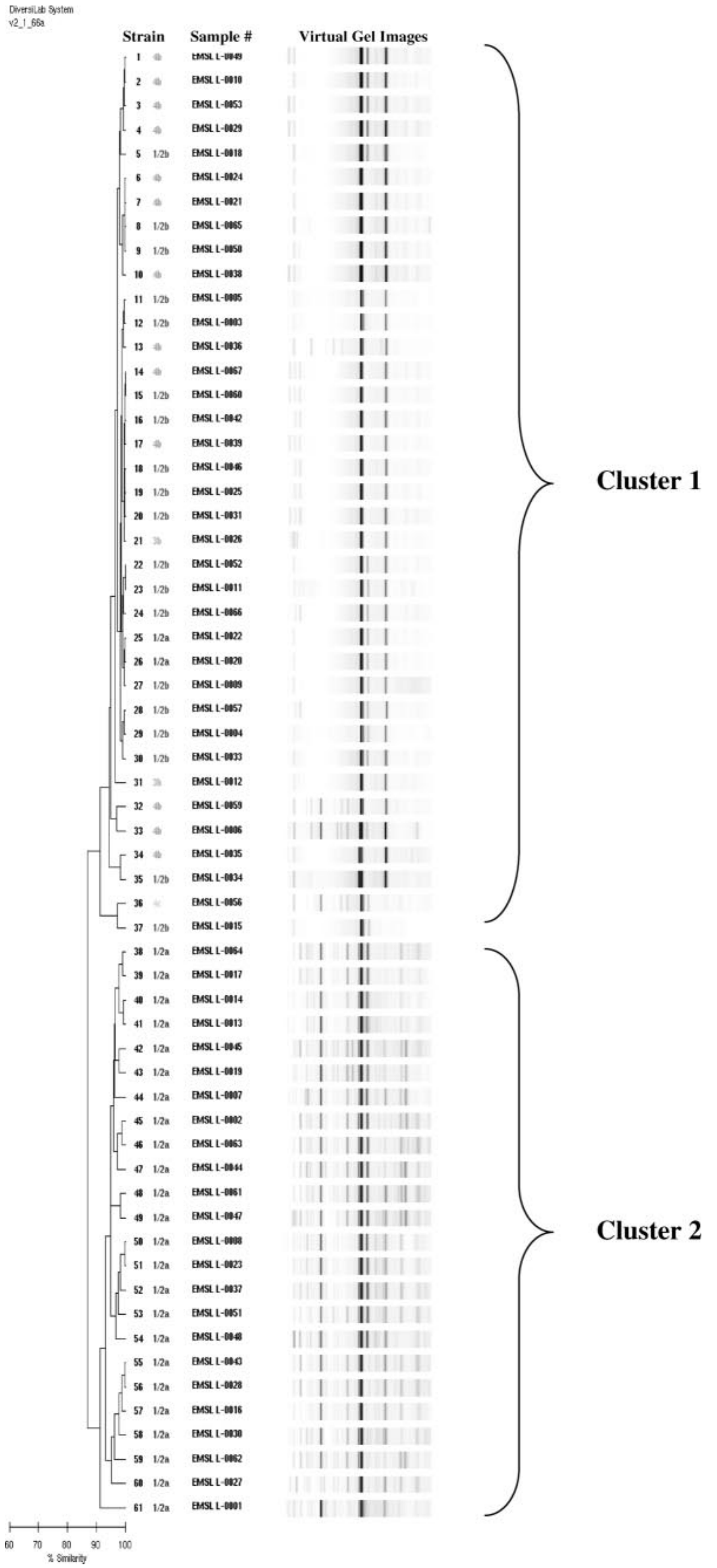


FIGURE 1. *Dendrogram and virtual gel images representing rep-PCR fingerprint patterns of* L. monocytogenes *isolates from raw milk.*

lates were grouped in the large, non-1/2a cluster (isolates 25 and 26; Fig. 1). These isolates were both from the same milk sample; one was isolated via direct culture, and the other was isolated after enrichment in selective medium. It is unclear why these isolates grouped separately from the other isolates of this serogroup.

Based on the dendrogram in Figure 2, *L. ivanovii* and *L. seeligeri* had less than 50% similarity with the *L. mon-*

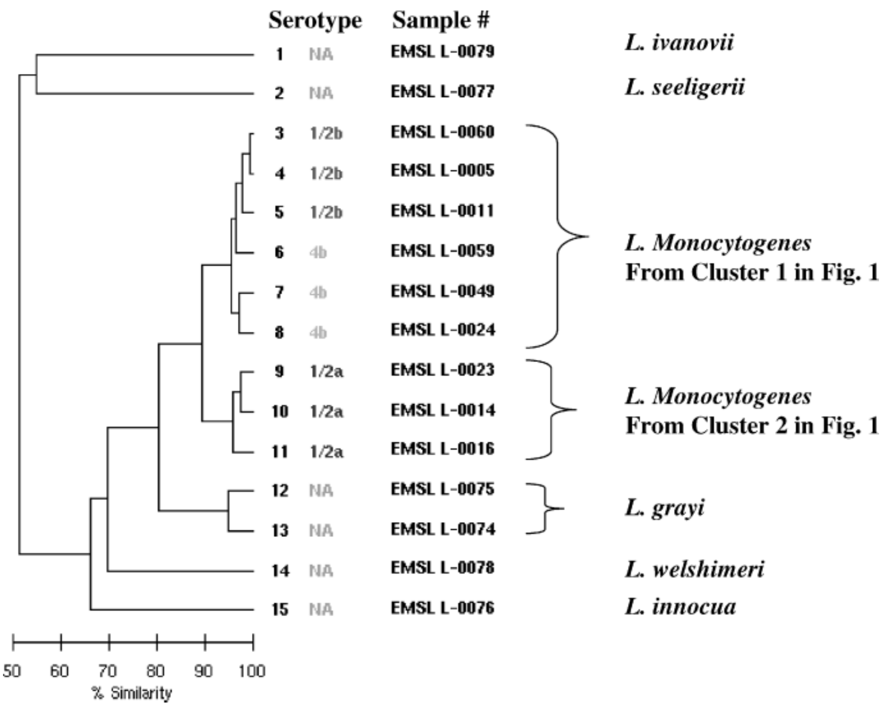


FIGURE 2. *Dendrogram representing rep-PCR fingerprints of selected* L. monocytogenes *isolates from raw milk and culture collection strains of* L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, *and* L. grayi.

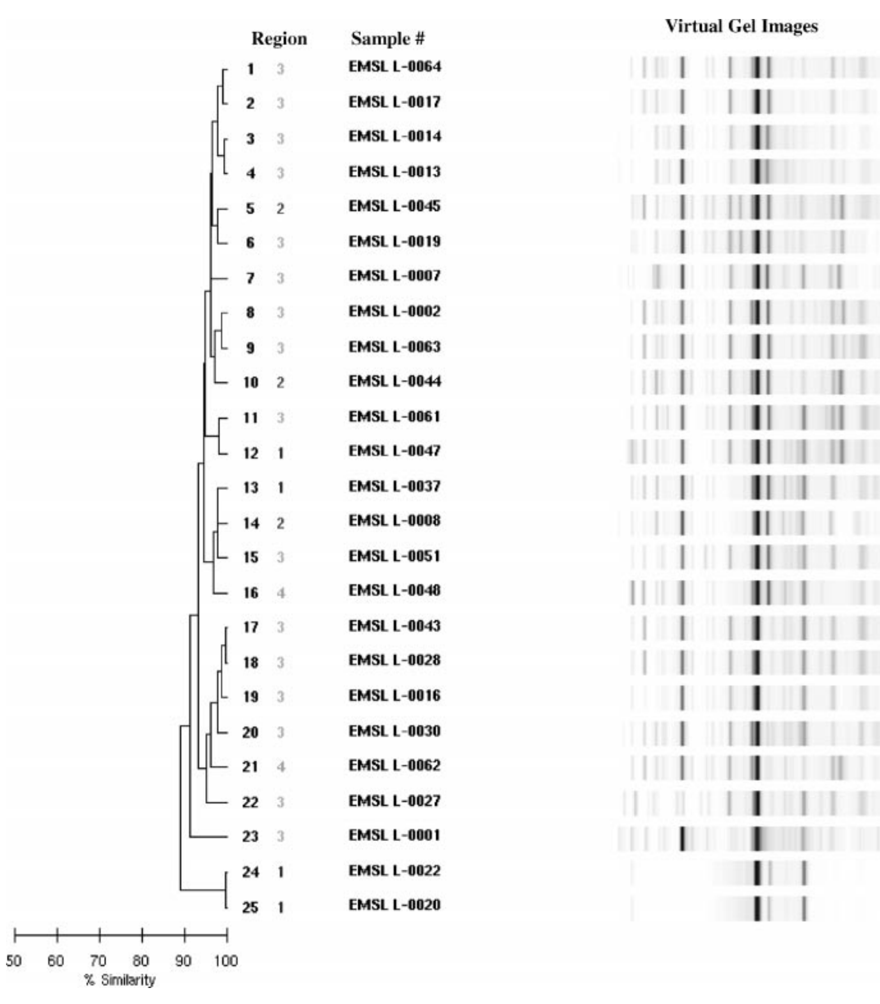


FIGURE 3. *Dendrogram and virtual gel images representing rep-PCR fingerprints of* L. monocytogenes *1/2a isolates from raw milk, with geographic origin of each strain: 1, west; 2, midwest; 3, northeast; 4, southeast.*

*ocytogenes* isolates in clusters 1 and 2. The remaining strains, *L. welshimeri, L. grayi,* and *L. innocua,* were more similar to the *L. monocytogenes* isolates of clusters 1 and 2, although the similarities were still very low (62 and 80%). Although additional isolates would be needed to build a database, it appears that this method may be useful for determining species once an isolate has been identified as *Listeria.*

As we reported previously *(21),* there were regional differences in serotype distribution of the *L. monocytogenes* strains used in this study. The strains were isolated from raw milk samples taken from dairy farms in 21 states. Serotype 4b was more prevalent in the southeast than in any of the other regions, serotype 1/2a was dominant in the northeast, serotype 1/2b was dominant in the midwest, and isolates from serogroups 1/2a and 1/2b were equally prevalent in the western region.

We were interested in determining whether we could identify specific regional distributions based on a more discriminating classification method. When rep-PCR fingerprints of the *L. monocytogenes* 1/2a isolates were compared by region (Fig. 3), there was no apparent regional grouping. The overrepresentation of isolates from the northeast (region 3) in comparison to the remaining three regions makes it difficult to draw conclusions regarding relationships among regions. However, the three isolates from the midwest (region 2) are highly dispersed among the isolates from the northeast. In this dendrogram, there are three sets of isolates that come from the same milk sample. In each case one isolate was obtained through direct culture, and the other was obtained after enrichment in selective medium. Two of the pairs (EMSL L-0013 and EMSL L-0014, and EMSL L-0020 and EMSL L-0022) are indistinguishable, and the other pair (EMSL L- 0016 and EMSL L-0043) has a very similar fingerprint pattern. These observations and the clustering and level of dissimilarity between isolates within this 1/2a serogroup suggest that this automated rep-PCR method might be useful for tracing *L. monocytogenes* 1/2a strains across geographic regions or within smaller ecological niches such as a dairy farm.

Although the automated rep-PCR method used in this study could be used to distinguish between species of *Listeria,* it could not discriminate between *L. monocytogenes* serotypes 1/2b and 4b. However, the method had sufficient discriminatory power to separate the majority of 1/2a isolates from the remaining *L. monocytogenes* serotypes. This method has the potential for discriminating between *L. monocytogenes* 1/2a isolates and may be useful in epidemiological studies of this serotype and as a complement to other methods of genomic subtyping.

## ACKNOWLEDGMENTS

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